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The Role of Arachidonic Acid and Eicosatrienoic Acids in the Activation of Spermatozoa in *Arenicola marina* L. (Annelida: Polychaeta)

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Abstract. Partial purification of a sperm maturation factor (SMF) in the intertidal polychaete *Arenicola marina* has implicated arachidonic acid, an arachidonic metabolite, or a similar substance as the active factor from the prostomium. The effects of a number of 20-carbon fatty acids on inactive spermatozoa are investigated, and this reveals that only arachidonic acid and 8,11,14-eicosatrienoic acid cause sperm activation. The use of argentation thin-layer chromatography to separate fatty acids with varying degrees of unsaturation reveals a component in prostomial lipid extract, which co-migrates with eicosatrienoic acids. Investigations using cyclooxygenase and lipoxygenase result in a loss of sperm-activating properties of both prostomial extract and fatty acids. The use of cyclooxygenase and lipoxygenase inhibitors has no effect. Bovine serum albumin (BSA) reduces the sperm activating properties of both fatty acids and prostomial extract in a dose-dependant way. Additional purification procedures using: (a) organic solvents and aqueous buffers and (b) ODS silica cartridges, demonstrate that the active fraction of prostomial extract co-elutes at every step with the 8,11,14-eicosatrienoic acid standard. Gas chromatography of methyl esters of prostomial lipid extract reveals the presence of a peak with an identical retention time to the methyl ester of authentic 8,11,14-eicosatrienoic acid standard. The results described here provide strong evidence that the active SMF in prostomial homogenate is not a fatty acid metabolite but the parent acid 8,11,14-eicosatrienoic acid. These results could only be made unequivocal by full structural analysis using mass spectrometry and NMR following capillary gas-liquid chromatography.

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Introduction

Arenicola marina is a common intertidal polychaete. Its reproductive cycle is annual, with most populations found around the coasts of the British Isles spawning in the autumn or early winter (Howie, 1959). The reproductive biology of this species has been reviewed by Howie (1984). In both sexes, gamete proliferation occurs in the gonads, and early germ cells are released into the coelomic fluid where gametogenesis proceeds (Ashworth, 1904; Newell, 1948). Females approaching maturity are characterized by many oocytes that have completed vitellogenesis but are arrested in prophase of meiosis I; maturing males have many sperm morulae (Howie, 1959). Sperm morulae are plates of several hundred fully differentiated immotile spermatozoa, which are bound together at both the head and distal ends of the flagella (Newell, 1948; Bentley 1985, 1986a, b; Bentley and Pacey, 1989).

Spawning in both male and female *Arenicola marina* is a direct consequence of the maturation of the gametes. The maturation of the oocytes (entry into metaphase of meiosis I), and the breakdown of the sperm morulae to free-swimming spermatozoa, results in the immediate shedding of these from the ciliated funnels of the nephromixia (Howie, 1961b, c). Oocytes mature by the action of a maturation hormone (Howie, 1963, 1966) from the prostomium, which induces germinal vesicle breakdown *in vitro* (Meijer and Durchon, 1977). The dissociation of the sperm morulae in males is also brought about

by a prostomial maturation hormone (sperm maturation factor) (Howie, 1963, 1966), which is a lipid (Howie, 1961a; Bentley, 1985).

Bentley (1985) began purifying the sperm maturation factor (SMF) using thin-layer chromatography, indicating that it was a relatively polar lipid. Lipids recovered from the TLC plates were tested for SMF activity in an *in vitro* assay. Biological activity was recovered from areas of the TLC plates where a number of pharmacologically active, non-steroid lipids are found. Further TLC studies led Bentley (1986a) to suggest that SMF may be a metabolite of the 20-carbon polyunsaturated fatty acid—arachidonic acid. Arachidonic acid (5,8,11,14-eicosatetraenoic acid) is one of a number, and probably the most important, of 20-carbon polyunsaturated fatty acids that are naturally occurring precursors of a wide range of extremely biologically active compounds. These include the prostaglandins, HETE's (hydroxy-eicosatetraenoic acids), and leukotrienes. Roles for these compounds have been identified in a wide range of vertebrates and invertebrates. Their roles in invertebrates have been recently reviewed (Stanley-Samuelson, 1987) and we will not discuss them further here. However, it should be noted that arachidonic acid is metabolized by starfish oocytes (Meijer and Guerrier, 1984; Meijer *et al.*, 1984; Meijer *et al.*, 1986), and that this results in the breakdown of the germinal vesicle prior to fertilization.

In light of the information available on the chemical nature of SMF, the present investigation examines in detail the possible role of arachidonic acid and the related 8,11,14-eicosatrienoic acid in the activation of spermatozoa in *Arenicola marina*.

Materials and Methods

Gravid individuals of *Arenicola marina* were collected by digging in sand during low water of spring tides at St. Andrews Bay, Fife, Scotland, and Fairlie Sands, Ayrshire, Scotland. Specimens were maintained individually in seawater at 5°C in the laboratory until use. Sperm samples for bioassay use were removed from the coelomic cavity as described previously (Bentley and Pacey, 1989). *In vitro* assays of sperm morula suspensions were performed as described by Bentley (1985).

Preparation of prostomial lipid extracts

Prostomial lipid extracts were prepared from mature specimens of *Arenicola marina*. The prostomia were removed using iridectomy scissors, and were homogenized using an MSE Soniprep 150 ultrasonic disintegrator at 0°C. The lipid fraction was partitioned from the sample using an equal volume of chloroform:methanol (2:1 v/v). The organic layer from several extractions was removed, pooled, and dried over anhydrous sodium sulphite. The samples were then concentrated by removing the solvent mixture in a rotary evaporator. The dried lipid residues were redissolved in methanol before being assayed for biological activity or used in subsequent analytical procedures.

Thin-layer chromatography (TLC)

A sample of total lipid was applied to 20 × 20 × 0.25 cm pre-coated silica gel F_{254} TLC plates (Merck) using a 100 μl disposable micropipette. Prior to applying the sample, the plates were cleaned of any lipid contaminants by running the blank plate, in the solvent system to be used, for its full length. After allowing the solvent to evaporate, the plate was activated in an oven at 120°C for 30 min. The solvent system used was the upper phase of: ethyl acetate:2,2,4-trimethylpentane:acetic acid:water (45:25:10:50 v/v) (Salmon and Flower, 1982) and the plates were run in a vertical chamber until the solvent front had moved 12 cm up the plate. The solvent was then allowed to evaporate from the plate in a fume cupboard, and the spots were visualized by spraying the plate with 10% phosphomolybdic acid in ethanol. A second plate was run simultaneously with the above, but was not sprayed with phosphomolybdic acid. Areas corresponding to the visualized spots on the first TLC plate were scraped off the plate and the lipids eluted in methanol and tested for biological activity as described above.

Argentation TLC

A sample of total prostomial lipid was spotted on to two further activated TLC plates impregnated with 5% $AgNO_3$ in acetone (see Christie, 1982). The plates were developed as for the TLC described above. Free fatty acid standards were also applied to the plates. When the solvent front had reached the 12-cm mark, the plates were removed, the solvent evaporated, and the plates washed with distilled water to remove the $AgNO_3$. One of the plates was sprayed with phosphomolybdic acid and the second plate was used for recovering the lipids for bioassay.

In vitro *assay of 20-carbon fatty acids*

Free fatty acids: eicosanoic, 11-eicosenoic, 11,14-eicosadienoic, 8,11,14-eicosatrienoic acid, 11,14,17-eicosatrienoic, 5,8,11,14-eicosatetraenoic (arachidonic), and 5,8,11,14,17-eicosapentaenoic acids, were obtained from Sigma Chemical Co., and 1×10^{-2} *M* stock solutions prepared in HPLC grade methanol (BDH). For use in bioassay, aliquots of these stock solutions were diluted 100 fold to give a final free acid concentration of 1×10^{-4} *M* and a negligible residual solvent concentration. Double dilutions of the free acids were then used to de-

termine the biological activity of each acid in the activation of spermatozoa *in vitro.*

Effect of cyclooxygenase and lipoxygenase pathway inhibitors

Stock solutions (10 m*M*) of the cyclooxygenase inhibitors aspirin, indomethacin, and tolazoline, were prepared in TFSW (triple filtered seawater). A 1-m*M* solution of butylated-hydroxytoluene, a lipoxygenase inhibitor, was also prepared. Prostomia were then homogenized in solutions of each inhibitor before bioassay for SMF activity. Control experiments were carried out in which the inhibitors of cyclooxygenase or lipoxygenase were added to prostomial extract after homogenization, or TFSW prior to bioassay. This permits the distinction to be made between metabolism of fatty acid substrate by the prostomial homogenate and metabolism by the spermatozoa themselves.

Incubation of biologically active fatty acid with cyclooxygenase and lipoxygenase

(A) Arachidonic acid was incubated with fresh bovine lung homogenate to obtain products from the cyclooxygenase pathway as described by Powell (1982). One gram of bovine lung tissue was homogenized on ice in 5 ml 0.05 *M* Tris-HCl buffer, pH 7.4. One ml of the homogenate was incubated with arachidonic acid at a final concentration of 1×10^{-2} *M* at 37°C for 5 min. The reaction was terminated by adding 5 ml ethanol, then adding 16 ml H_2O, and centrifuging at $400 \times g$ for 10 min. The supernatant was removed and assayed for biological activity.

(B) One-ml aliquots each containing 1.8 mg (*c.* 250,000 units) of soybean lipoxygenase was incubated with arachidonic acid at a final concentration of 5×10^{-3} *M* at 25°C for 15 min. After incubation, the reaction was terminated by heating, the extract was centrifuged, and the supernatant was assayed for biological activity. Incubations containing denatured lipoxygenase and lacking lipoxygenase were also carried out.

Incubation of prostomial extract with lipoxygenase

One-ml aliquots each containing the equivalent of 0.36 prostomium were incubated with 1.8 mg (*c.* 250,000 units) of lipoxygenase, for 60 min at 20°C. After incubation, the reaction was stopped and the sample treated as described above. Incubations containing denatured lipoxygenase and lacking lipoxygenase were carried out in parallel.

Incubations of biologically active fatty acid with BSA

Prostomial homogenate and 8,11,14-eicosatrienoic acid were bioassayed in the presence of dissolved BSA (bovine serum albumin). BSA solutions were freshly prepared in TFSW to give a final concentration in the assay of 0, 100, 1000 $\mu g \cdot ml^{-1}$, and 10 $mg \cdot ml^{-1}$, respectively.

Extraction of SMF by organic solvents and aqueous buffers

Extraction of SMF from biologically inactive lipid constituents of prostomial extracts were carried out as described by Jouvenaz *et al.* (1970) and Van Dorp (1971). This allows larger quantities of starting material to be purified, and permits the separation of free fatty acids from their often biologically active, but extremely labile, metabolites. This procedure involves the initial preparation of ethanolic lipid extract, washing the residue with ethanol:diethyl ether (1:1 v/v), followed by adding saline. The extract is then reduced in volume to 2.5 ml, acidified to pH 4 with citric acid, and contaminating lipids removed with petroleum ether. The remaining lipids are then taken up into ethyl acetate concentrated to about 5 ml total volume. Tris buffer (1.5 ml, pH 7.8) is then added to take up any prostaglandins present. All the organic and aqueous fractions obtained throughout this procedure were bioassayed for SMF activity.

Extraction of SMF on ODS silica cartridges

Freshly prepared prostomial homogenate and free fatty acid standards were separately applied to pre-wet Sep-Pak® C_{18} Cartridges (Waters Associates) in 10% aqueous ethanol with the pH adjusted to 4.0 using a 1-*M* stock solution of citric acid. The Sep-Pak® was prewetted using 2 ml of methanol followed by 5 ml of H_2O before applying the sample or standard. Fractions were partitioned using the following solvent mixtures (Powell, 1982): aqueous ethanol (20 ml, 10%), 20 ml H_2O, 10 ml petroleum ether, 10 ml petroleum ether:chloroform (65:35 v/v), and 10 ml methyl formate. The Sep Pak® was regenerated using 10 ml of 80% aqueous ethanol. The fraction obtained using each solvent was collected and prepared for bioassay as described above.

Gas-liquid chromatography of prostomial lipids

Prostomial lipid extracts were prepared as described above, and methylated using a method modified from Christie (1982). The sample was dissolved in 1 ml of dichloromethane, and refluxed for 2 h with 2% methanolic H_2SO_4. After cooling, 4 ml of saturated NaCl was added, and the fatty acid methyl esters extracted with 2 ml petroleum ether (40°–60°C). GC analysis was performed using a Hewlett Packard 5890A gas chromatograph fitted with a flame ionisation detector. Samples were separated on a capillary non-polar column (fused silica, 25 m $\times$ 0.25 mm i.d., 0.12 df, CP-Sil 5CB) following on-col-

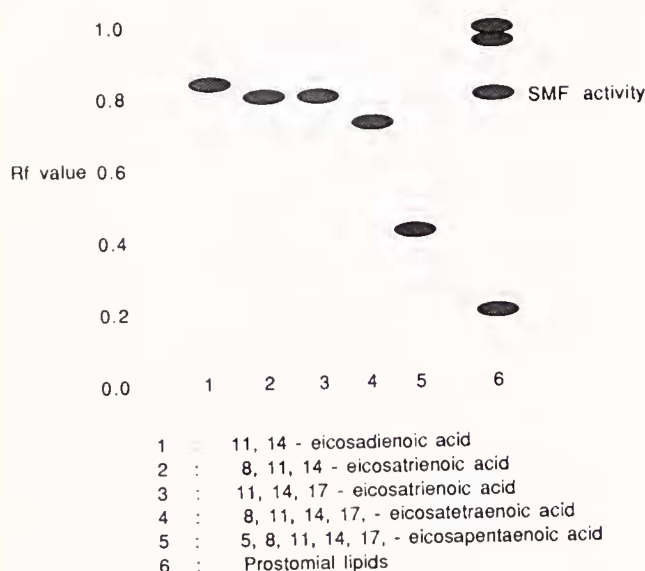

Figure 1. Separation of 20-carbon fatty acids (spots 1 to 5 with 2, 3, 3, 4, and 5 double bonds, respectively) and prostomial lipids by argentation thin-layer chromatography. The fatty acid standards with the greatest degree of saturation interact least with the silver nitrate on the TLC plate and therefore migrate furthest. The prostomial lipid extract shows a spot with an identical Rf value to the eicosatrienoic acids. This spot, when scraped off a washed plate, shows sperm maturation factor (SMF) activity *in vitro*.

umn injection. A linear thermal gradient program from 90°–300°C at 20°C·min^{-1} was used with helium as the carrier gas (25 cm·s^{-1}).

Results

Analysis of prostomial lipid extract and C20 fatty acids by TLC

TLC on activated silica gel plates, using the upper phase of: ethyl acetate:2,2,4-trimethylpentane:acetic acid:water (45:25:10:50 v/v) as a solvent, allows the separation of C20 fatty acids from their metabolites. The biological activity associated with prostomial lipid samples is associated with a region of the TLC plate that is identical to the position where free fatty acids are found (Rf 0.78–0.82). This chromatographic separation cannot distinguish between fatty acids with varying degrees of unsaturation.

Argentation TLC (using the same solvent system and TLC plates impregnated with 5% $AgNO_3$) allows fatty acids of the same carbon number to be separated according to the number of double bonds in the molecule. Figure 1 illustrates the results of this separation. SMF activity was recovered from a region of the plate which corresponds to the position of the C20:3 acids (8,11,14-eicosatrienoic acid and 11,14,17-eicosatrienoic acid). This technique is unable to separate these two isomers because they are identical in their degree of unsaturation.

Sperm activation by C20 fatty acids

In vitro bioassay of eicosanoic, 11-eicosenoic, 11,14-eicosadienoic, 8,11,14-eicosatrienoic acid, 11,14,17-eicosatrienoic, 5,8,11,14-eicosatetraenoic (arachidonic), and 5,8,11,14,17-eicosapentaenoic acids for the ability to induce sperm activation showed that both 8,11,14-eicosatrienoic acid and arachidonic acid displayed biological activity. The results are summarized in Table I. Concentration ranges for biological activity of 8,11,14-eicosatrienoic acid and arachidonic acid are based on nine replicate experiments producing mean minimum concentrations required for a response of 4.47×10^{-5} *M* and 2.28×10^{-4} *M*, respectively. These data indicate that 8,11,14-eicosatrienoic acid is about five times more active in this system than in arachidonic acid.

Studies of cyclooxygenase and lipoxygenase pathways

The preparation of prostomial extract in the presence of inhibitors of cyclooxygenase activity (aspirin, indomethacin, tolazoline) or lipoxygenase (butylated hydroxytoluene) did not effect the SMF activity of the extract. Aspirin at concentrations of 5 m*M* and 10 m*M* caused sperm lysis (the reasons for this are not clear, but this effect is unlikely to be related to the cyclooxygenase inhibitory property of the aspirin). These results suggest that there is no conversion of parent fatty acid to a biologically active metabolite via either the cyclooxygenase or lipoxygenase pathway. However, polychaete enzymes metabolizing fatty acids may differ from vertebrate cyclooxygenases and lipoxygenases, and substances used as inhibitors may not effect enzyme activity. Quercetin, another lipoxygenase inhibitor, could not be used in this

Table I

Sperm activation by C20 fatty acids

Fatty acid	Activity	Threshold concentration for activation (Mean ± S.E.; n = 9)
A eicosanoic	−	
B 11-eicosenoic	−	
C 11,14-eicosadienoic	−	
D 8,11,14-eicosatrienoic	+	$4.47 \pm 1.46 \times 10^{-5}$ *M*
E 11,14,17-eicosatrienoic	−	
F 5,8,11,14-eicosatetraenoic	+	$2.28 \pm 1.78 \times 10^{-4}$ *M*
G 5,8,11,14,17-eicosapentaenoic	−	
H TFSW control	−	

Table II

The effects of cyclooxygenase and lipoxygenase on sperm activation by C20 fatty acids

	Activity	Threshold concentration for activation
a. Incubation with cyclooxygenase		
Arachidonic acid incubated with bovine lung cyclooxygenase	−	
Arachidonic acid	+	$1.25 \times 10^{-5}\ M$
Bovine lung homogenate (cyclooxygenase)	−	
TFSW	−	
b. Incubation with lipoxygenase		
Arachidonic acid incubated with soybean lipoxygenase	+	$2.5 \times 10^{-3}\ M$
Arachidonic acid	+	$4.0 \times 10^{-5}\ M$
Soybean lipoxygenase	−	
TFSW	−	

study because of a non-specific effect on spermatozoa, which will be reported elsewhere.

Incubation of arachidonic acid with bovine lung homogenate (cyclooxygenase) or soybean lipoxygenase was carried out to examine whether there was (a) a reduction, (b) enhancement, or (c) the same level of sperm activation after converting the fatty acid substrate to metabolites. Table II shows that both incubation with bovine lung homogenate and soybean lipoxygenase brought about a reduction in the fatty acid incubate's ability to activate spermatozoa. This suggests that the fatty acid has been largely converted to cyclooxygenase and lipoxygenase metabolites, which cannot activate the spermatozoa. Thin layer chromatographic analysis of the incubates confirm that most of the fatty acid is converted during incubation (Fig. 2). Thin layer chromatography of prostomial homogenate shows that most of the fatty acid remains unmetabolized. This indicates that the fatty acid is not normally converted to a metabolite by endogenous polychaete enzymes, which may be outcompeted for substrate during incubations with exogenous cyclooxygenase or lipoxygenase.

Incubation of prostomial homogenate with soybean lipoxygenase results in a total loss of SMF activity. This indicates the conversion of a fatty acid in the prostomial homogenate to non-active metabolites, and also suggests that it is this fatty acid component of the prostomial homogenate that causes sperm activation *in vitro*.

Incubations of 8,11,14-eicosatrienoic acid and prostomial extract with BSA

BSA (bovine serum albumin) was incubated with 8,11,14-eicosatrienoic acid and prostomial homogenate to investigate the possible interference of BSA on the ability of both the fatty acid and prostomial extract to activate spermatozoa of *Arenicola marina*. It has long been known that fatty acids interact strongly with serum albumin (Goodman, 1958). Figure 3 shows that the ability of both 8,11,14-eicosatrienoic acid and prostomial homogenate to activate spermatozoa is markedly reduced by the addition of BSA. This evidence lends further support to the suggestion that it is a fatty acid component of the prostomial homogenate that causes sperm activation *in vitro*.

Further purification of SMF from prostomial homogenate

Figure 4 shows the purification steps employed for the purification of SMF from crude prostomial homogenate, using the method developed by Jouvenaz *et al.*, (1970) and Van Dorp (1971) for the extraction of prostaglandins from biological tissues. The figure also traces the biological activity through the purification steps. SMF activity is finally recovered in an ethyl acetate fraction.

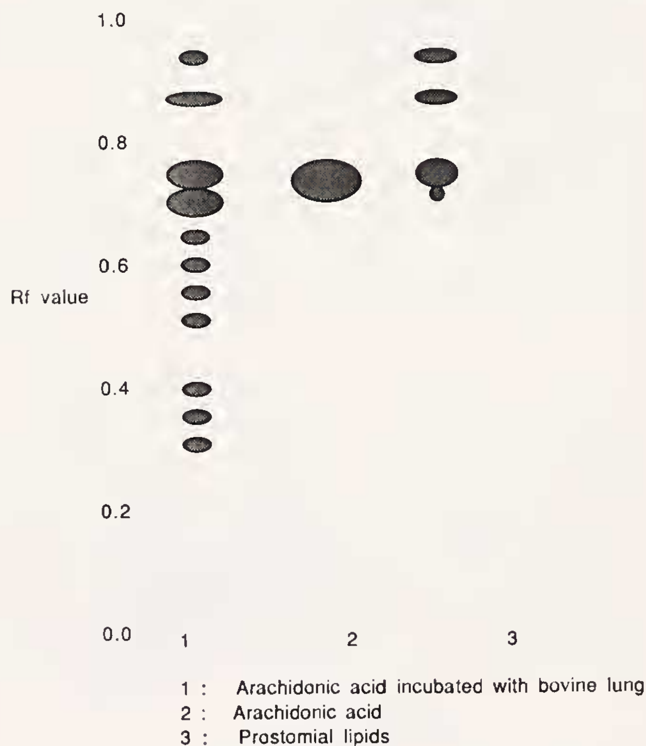

Figure 2. TLC analysis of prostomial lipids, arachidonic acid, and arachidonic acid following incubation with cyclooxygenase. Arachidonic acid can be seen at an Rf value of about 0.78. The cyclooxygenase products are clearly visible with Rf values lower than that of arachidonic acid itself. Prostomial lipid extract shows no spots which correspond to cyclooxygenase products, and which may have arisen as a result of action by endogenous cyclooxygenases.

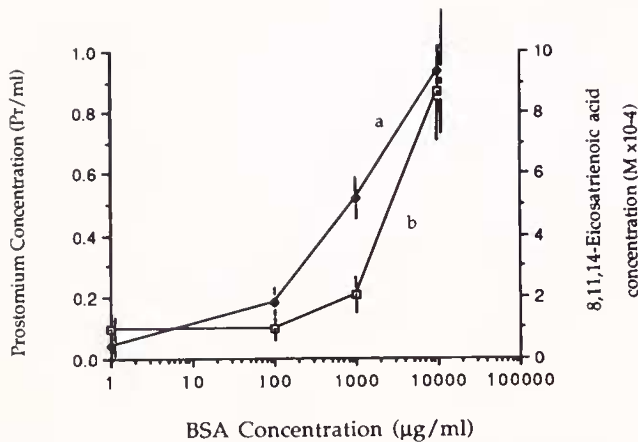

Figure 3. Minimum concentrations of (a) 8,11,14-eicosatrienoic acid, and (b) prostomial homogenate required to bring about sperm activation in the presence of bovine serum albumin (BSA). The fatty acid concentration, or the concentration of prostomial extract required to bring about sperm activation *in vitro*, increases with the concentration of dissolved BSA. Data shown are the mean (±SE) minimum concentrations required to bring about sperm activation in three replicated experiments.

Prostaglandins remain in the pH 7.8 Tris buffer and would be recovered only in an ethyl acetate fraction from Tris buffer at pH 4.0. This indicates that SMF activity is not recovered with prostaglandins but is recovered in the fatty acid fraction.

A parallel approach to the purification of SMF has been carried out using Sep-Pak® cartridges and a succession of aqueous and organic solvents. Table III shows that SMF activity is recovered in the same fractions as the 8,11,14-eicosatrienoic acid standard.

Gas chromatographic analysis of prostomial lipids

The results of separation of methyl esters of prostomial lipid extracts are shown in Figure 5. Three peaks with retention times corresponding to methyl esters of 5,8,11,14-eicosatetraenoic acid (8.72 min), 8,11,14-eicosatrienoic acid (8.81 min), 11,14,17-eicosatrienoic acid (8.93 min), can be identified. This clearly indicates the presence of 8,11,14-eicosatrienoic acid in prostomial lipid extracts obtained from prostomia showing SMF activity *in vitro*.

Discussion

The results obtained from thin layer chromatography of prostomial total lipid extracts described above showed that SMF activity co-migrated with 20-carbon fatty acid standards. In particular, it is associated with eicosatrienoic acids (demonstrated by argentation TLC). The bioassay of C20 fatty acids show that only two of the fatty acids tested brought about the activation (dissociation of the morulae and the acquisition of motility) *in vitro*: arachidonic and 8,11,14-eicosatrienoic acids. Arachidonic acid, while capable of activating spermatozoa, does not co-migrate with prostomial SMF in the argentation TLC. Clearly, then, arachidonic acid and SMF are not the same substance. All eicosatrienoic acids co-migrate in this TLC system but only 8,11,14-eicosatrienoic acid causes sperm activation *in vitro*. While 8,11,14-eicosatrienoic acid co-migrates with SMF, and has biological activity identical to SMF, this is insufficient evidence to propose that they are the same.

Arachidonic acid, 8,11,14-eicosatrienoic acid, and eicosapentaenoic acid are all naturally occurring 20-carbon fatty acids that differ in the number of double bonds, having 4, 3, and 5 double bonds, respectively. They are all precursors for a range of pharmacologically active molecules, the eicosanoids. Each of these three fatty

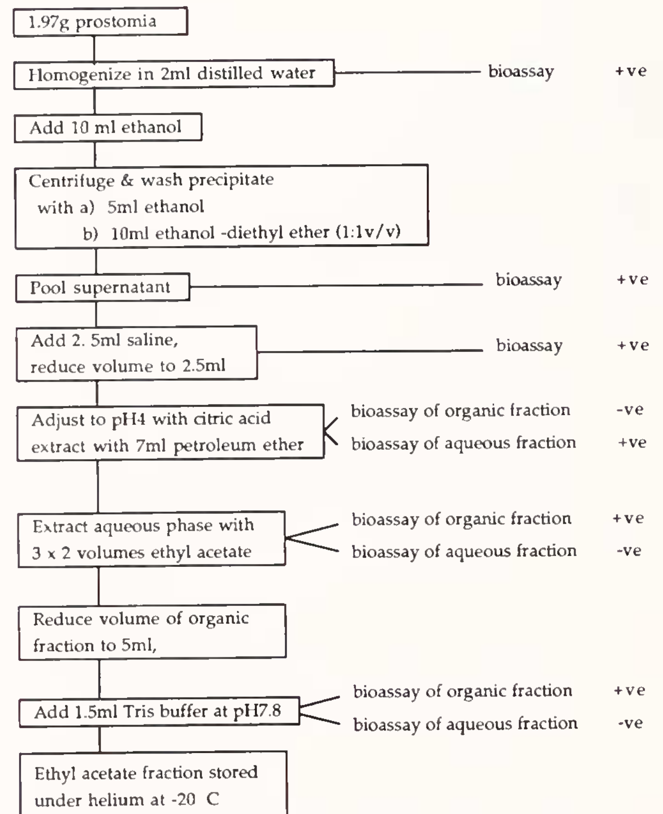

Figure 4. Extraction procedure used for sequential purification of sperm maturation factor (SMF) using organic solvents and aqueous buffers (after Jouvenaz *et al.*, 1970). Following each purification step, aqueous and organic phases were dried under helium, resuspended, and tested for their ability to activate sperm *in vitro*. The response is represented here as +ve or −ve, where +ve indicates the presence of SMF activity evidenced by sperm morula breakdown and the presence of free-swimming spermatozoa, and −ve indicates no activation of spermatozoa.

Table III

Purification of prostomial SMF on ODS silica cartridges

Eluent from cartridge	Activity of prostomium extract	Activity of 8,11,14-eicosatrienoic acid
1. 20 ml 30% ethanol	–	–
2. 20 ml H_2O dist.	–	–
3. 10 ml petroleum ether:chloroform (65:35 v/v)	+	+
4. 10 ml methyl formate	+	+
5. 10 ml 80% ethanol	–	–

acids gives rise to a series of prostaglandins (PGs): arachidonic acid, which is the best known and probably the most important, is converted to series 2 PGs; 8,11,14-eicosatrienoic acid is converted to series 1 PGs. Eicosapentaenoic acid, which is the most important C20 fatty acid in marine organisms, gives rise to series 3 PGs.

The use of the principal enzymes involved in the metabolism of the fatty acids to their respective prostaglandins (cyclooxygenase) and other metabolites (lipoxygenase) combined with the use of selective inhibitors permits the possible pathways involved to be elucidated. Evidence shown above, by using bovine lung homogenate and soybean lipoxygenase, which both caused a marked reduction of SMF activity of prostomial homogenate, suggests strongly that a fatty acid present in prostomial homogenate is responsible for the SMF activity. The use of inhibitors of cyclooxygenase and lipoxygenase suggests that there is no conversion of fatty acid in prostomial homogenate to metabolite(s), which may have potent biological activity as previously suggested (Bentley, 1986a).

Compared to other invertebrate groups, notably the insects, and the Crustacea, little is known of the chemical nature of polychaete hormones. To date, no hormone from polychaete tissues has been completely purified or its structure elucidated. Grothe *et al.* (1987) identified catecholamines in the nervous system of *Ophryotrocha puerilis*, which may have an endocrine related function. Numerous vertebrate-like peptides have been identified in the nervous system of polychaetes (Dhainaut-Courtois *et al.*, 1985), but functions have yet to be ascribed to these putative hormones. The possible action of a fatty acid as a hormone may seem unlikely,

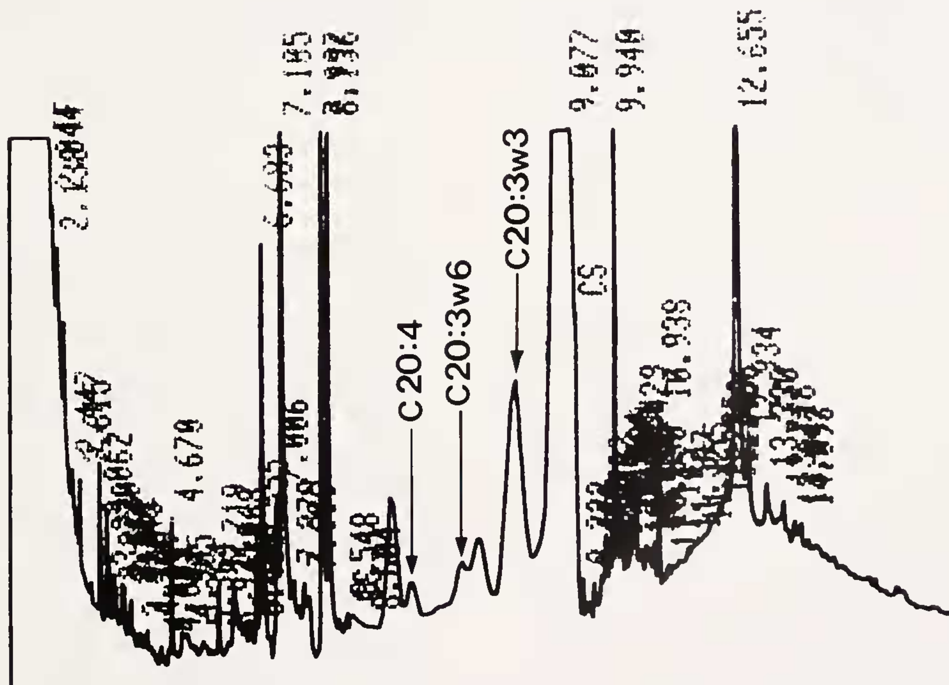

Figure 5. Gas chromatograph of fatty acid methyl esters (FAMEs) of biologically active prostomial lipids showing identified peaks corresponding to 5,8,11,14-acid (C20:4), 8,11,14-eicosatrienoic acid (C20:3w6), and 11,14,17-eicosatrienoic acid (C20:3w3) with retention times of 8.72 min, 8.81 min, and 8.93 min, respectively. These peaks correspond in absolute retention times, and relative distances to FAME standards of the three acids.

but the certain presence in the prostomium of *Arenicola marina* of a fatty acid that acts on distant target cells (sperm morulae in the coelomic fluid) may well be an example of such a "hormone." Its hormonal role is further supported by the cyclical nature of its appearance in prostomial extracts. Bentley (1985) showed that SMF activity of prostomial extracts is maximal around the breeding season of given populations and is non-existent during the post-spawning period.

Fatty acids and prostaglandins are present in a wide range of lower animals (Srivastava and Mustafa, 1984; Stanley-Samuelson, 1987). These essential fatty acids and their metabolites also have an effect on many aspects of reproduction in marine invertebrates. The endocrine control of oocyte maturation in asteroid echinoderms is now well understood and involves the action of a peptide gonad-stimulating substance (GSS), 1-methyl adenine, and an intracellular maturation-promoting factor (MPF) (see Giese and Kanatani, 1987 for review). One-methyl adenine acts on the oocytes to bring about maturation. However, Meijer *et al.* (1986) demonstrated that arachidonic acid mimics the action of 1-methyl adenine on starfish oocytes *in vitro*. It is possible that 1-methyl adenine acts as a "second messenger" or that the arachidonic acid mimics some hitherto unidentified fatty acid.

A tri-hydroxy metabolite of arachidonic acid has been identified as the hatching factor in the barnacle *Semibalanus* (*Balanus*) *balanoides* (Clare *et al.*, 1982, 1985; Holland *et al.*, 1985). Prostaglandins also cause spawning of the abalone, *Haliotis rufescens*, and the mussel, *Mytilus edulis* (Morse *et al.*, 1977).

Pharmacologically active metabolites of arachidonic and related fatty acids are characteristically short-lived substances produced close to, or at, their site of action. The parent fatty acids are often metabolized by the target cells themselves. This may occur at the cell surface or intracellularly. Typically this metabolism occurs as a result of the action of lipoxygenases or cyclooxygenase (PG synthetase). The precise nature of the enzymes may vary between phyla, and there is evidence that those found in some invertebrates (*e.g.*, *Lymnaea stagnalis*) may be different to those occurring in vertebrates (Clare *et al.*, 1986). In the starfish oocyte, arachidonic acid is converted to HETEs at the plasma membrane (Meijer *et al.*, 1986). It may be that 8,11,14-eicosatrienoic acid is metabolized by the sperm morulae of *Arenicola marina* and this will be investigated by the use of radiolabeled precursors. The maturation of starfish oocytes by arachidonic acid is inhibited in a dose-dependent manner by the presence of BSA, however, maturation induced by 1-methyl adenine (the natural inducer) is not. The activation of spermatozoa of *A. marina* by prostomial extract or 8,11,14-eicosatrienoic acid are both inhibited in a similar dose-dependent manner by BSA. This may be further evidence to suggest that the fatty acid from the prostomium, causing sperm activation in *A. marina*, is a primary inducer rather than a "second messenger."

Purification procedures, followed by structural analysis, must be performed to identify the chemical nature of any endocrine substance with certainty. For example, the barnacle hatching factor was identified by organic extraction and subsequent GC-MS analysis (Holland *et al.*, 1985). One of the problems often encountered is obtaining sufficient starting material for purification. The separation procedures described in this paper show that SMF of *Arenicola marina* has identical chromatographic properties to 8,11,14-eicosatrienoic acid, and that 8,11,14-eicosatrienoic acid is present in the fatty acid component of prostomial extract. The use of bonded-phase C18 cartridges as a purification stage should permit sufficient quantities of SMF to be extracted to complete mass spectrometrical analysis.

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